

# Mutation analysis of the cross-reactive epitopes of Japanese encephalitis virus envelope glycoprotein

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Group and serocomplex cross-reactive epitopes have been identified in the envelope (E) protein of several flaviviruses and have proven critical in vaccine and diagnostic antigen development. Here, we performed site-directed mutagenesis across the E gene of a recombinant expression plasmid that encodes the Japanese encephalitis virus (JEV) premembrane (prM) and E proteins and produces JEV virus-like particles (VLPs). Mutations were introduced at I135 and E138 in domain I; W101, G104, G106 and L107 in domain II; and T305, E306, K312, A315, S329, S331, G332 and D389 in domain III. None of the mutant JEV VLPs demonstrated reduced activity to the five JEV type-specific mAbs tested. Substitutions at W101, especially W101G, reduced reactivity dramatically with all of the flavivirus group cross-reactive mAbs. The group and JEV serocomplex cross-reactive mAbs examined recognized five and six different overlapping epitopes, respectively. Among five group cross-reactive epitopes, amino acids located in domains I, II and III were involved in one, five and three epitopes, respectively. Recognition by six JEV serocomplex cross-reactive mAbs was reduced by amino acid substitutions in domains II and III. These results suggest that amino acid residues located in the fusion loop of E domain II are the most critical for recognition by group cross-reactive mAbs, followed by residues of domains III and I. The amino acid residues of both domains II and III of the E protein were shown to be important in the binding of JEV serocomplex cross-reactive mAbs.

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## INTRODUCTION

Serological testing completed in the early 20th century classifies arboviruses into groups A and B by serological cross-reactivity. Japanese encephalitis virus (JEV) and other flaviviruses fall into group B (Casals & Brown, 1954). Japanese encephalitis is the leading cause of encephalitis in humans in Asian countries (Vaughn & Hoke, 1992). JEV belongs to the family *Flaviviridae* and has a positive-sense ssRNA genome of approximately 11 000 nt in length (Chambers *et al.*, 1990; Lindenbach & Rice, 2003). Recent history has seen a global resurgence of diseases caused by arboviruses once thought to be under control, such as JEV, tick-borne encephalitis virus (TBEV) and yellow fever virus, as well as viruses with expanded geographical distribution and disease severity, such as West Nile virus (WNV) and dengue virus serotypes 1–4 (DENV-1 to -4) (Lanciotti *et al.*, 1999; Mackenzie *et al.*, 2004). Co-circulating flaviviruses and

compulsory immunization confound serodiagnosis and can impact pathogenesis (Halstead, 1988).

The flavivirus envelope (E) glycoprotein contains three structural and functional domains (Kanai *et al.*, 2006; Rey *et al.*, 1995). E domain I (EDI) contains predominantly type-specific non-neutralizing (non-NT) epitopes and is an eight-stranded  $\beta$ -barrel. E domain II (EDII) contains cross-reactive epitopes eliciting both weakly NT and non-NT antibodies and contains the highly conserved internal fusion peptide (FP). E domain III (EDIII) has an Ig-like structure and contains the majority of type-specific, potentially NT epitopes as well as the primary receptor-binding motifs (Beasley & Barrett, 2002; Crill & Chang, 2004; Kanai *et al.*, 2006; Oliphant *et al.*, 2006; Rey *et al.*, 1995; Roehrig *et al.*, 1983, 1989, 1990, 1998; Stiasny *et al.*, 2006). Flavivirus infections elicit antibodies that cross-react with other members of the flavivirus group, as well as antibodies that cross-react with other viruses within the same serocomplex. Recently, amino acids located in the highly conserved E FP of EDII, particularly W101, G104,

A supplementary figure and table are available with the online version of this paper.

**Table 1.** Type-specific mAb reactivity against WT and mutant VLPs

mAb end-point reactivity of WT and mutant VLPs was determined via Ag-ELISA and is expressed on a logarithmic scale. ND, Not determined. Bold type indicates a mutant VLP Ag-ELISA end-point titre  $>4$ -fold that of WT VLPs.

VLP	MHIAF	Type-specific mAbs (reciprocal log <sub>10</sub> end-point titre)				
		112	503	2F2	2H4	J3 14 H5-2
WT	5.39	5.39	3.95	5.86	5.86	3.00
W101G	5.39	4.91	3.95	5.86	5.86	ND
W101K	5.40	<b>6.00</b>	3.95	ND	ND	ND
W101E	5.40	<b>6.00</b>	3.95	ND	ND	ND
W101F	5.40	$\geq 6.40$	3.95	ND	ND	ND
G104H	5.40	ND	ND	5.86	5.86	$<3$
G106K	5.40	5.86	3.95	<b>6.40</b>	5.86	3.00
L107D	5.40	5.86	3.95	5.86	5.86	<b>3.90</b>
I135S	5.40	ND	ND	5.86	5.86	3.00
E138K	5.90	ND	ND	5.86	5.86	3.00
T305E	5.40	<b>6.00</b>	3.95	ND	ND	ND
T305H	5.40	<b>6.00</b>	3.95	ND	ND	ND
E306G	5.41	ND	ND	$\geq 5.71$	$\geq 5.71$	ND
K312E	5.86	5.39	3.95	<b>6.40</b>	5.86	ND
A315E	5.40	<b>6.00</b>	3.95	ND	ND	ND
A315R	5.40	5.40	3.95	ND	ND	ND
S329A	5.41	ND	ND	5.71	5.71	ND
S331K	5.41	ND	ND	5.71	5.41	ND
G332R	5.41	ND	ND	5.71	5.41	ND
D389G	5.41	ND	ND	5.71	5.41	ND

G106 and L107, have been identified as important flavivirus group cross-reactive epitope determinants in DENV-2, TBEV, St Louis encephalitis virus, WNV and JEV (Chiou *et al.*, 2008; Crill & Chang, 2004; Lai *et al.*, 2008; Oliphant *et al.*, 2006; Stiasny *et al.*, 2006; Trainor *et al.*, 2007).

Complex cross-reactive epitopes were found to contain residues in EDIII, including K305, K307, L308, E309, K310, E311, V312, L387, L389 and W391, in DENV-2 and -3 (Crill *et al.*, 2009; Gromowski *et al.*, 2008; Matsui *et al.*, 2009; Sukupolvi-Petty *et al.*, 2007). Here we present results from mutagenesis of the JEV E protein and subsequent epitope mapping utilizing murine mAb panels to identify and ablate group and serocomplex cross-reactive epitopes. Our mAb epitope mapping results identify five subtly different group cross-reactive epitopes located in the EDIIFP, centred on W101, and six different complex cross-reactive epitopes, five of which are centred in the A sheet and BCx loop of EDIII, but also include residues in the EDIIFP.

## RESULTS

### mAb reactivity and epitope characterization

Epitope mapping of the JEV wild-type (WT) and mutant virus-like particles (VLPs) was carried out using a collection of five JEV-specific, six group cross-reactive and seven complex cross-reactive murine mAbs (Tables 1, 2 and 3). An

antigen-capture ELISA (Ag-ELISA) was used to standardize VLP concentrations and then to characterize individual VLP reactivity with the mAb panel.

End-point titres for five JEV type-specific mAbs, 112, 503, 2F2, 2H4 and J13 14 H5-2, against JEV WT VLPs ranged from  $10^{3.00}$  to  $10^{5.86}$  (Table 1). None of the JEV type-specific mAbs examined demonstrated reduced reactivity with any of the mutant JEV VLPs. In contrast, some of these mAbs experienced enhanced reactivity with mutant VLPs compared with WT (e.g. mAb 112 with W101, T305 and A315 substitutions). These data suggest that some substitutions not only maintain VLP recognition by JEV type-specific mAbs, but increase their reactivity.

Six flavivirus group cross-reactive mAbs, 6B6C-1, 4G2, 23-1, 23-2, 6B3B-3 and 5-2, reacted with WT JEV VLP, with titres ranging from  $10^{4.43}$  to  $\geq 10^{6.82}$  (Table 2). Substitutions at W101, especially W101G, reduced reactivity dramatically with all six flavivirus group-reactive mAbs. The reactivity of mAbs 6B6C-1, 4G2 and 5-2 was also reduced by the G104H and G106K substitutions, and the reactivities of all group cross-reactive mAbs except 6B6C-1 were reduced by the L107D substitution. Of the EDI substitutions, only E138K reduced reactivity with mAb 5-2. The reactivity of 6B6C-1, 4G2 and 6B3B-3 was additionally reduced by substitutions T305E, K312E, A315E, A315R, S329A, S331K, G332R and D389G. Unexpectedly, the reactivity of 6B6C-1 was enhanced against VLPs containing mutations L107D, I135S or E138K. These results suggest that, whilst amino acid residues of EDII are most critical for group cross-reactive mAbs,

**Table 2.** Group cross-reactive mAb reactivity against WT and mutant VLPs

mAb end-point reactivity of WT and mutant VLPs was determined via Ag-ELISA and is expressed on a logarithmic scale. ND, Not determined. Underlining indicates a mutant VLP Ag-ELISA end-point titre reduced by >4-fold that of WT VLPs; bold type indicates a mutant VLP Ag-ELISA end-point titre increased by >4-fold that of WT VLPs.

VLPs	Group cross-reactive mAbs (reciprocal log <sub>10</sub> end-point titre)					
	6B6C-1	4G2	23-1	23-2	6B3B-3*	5-2*
WT	5.39	6.40	≥ 6.82	5.39	5.86	4.43
W101G	<3	<3	<3	<3	<3	<3
W101K	<3	5.20	3.00	3.95	<3	<3
W101E	<3	5.20	3.00	<3	<3	<3
W101F	3.95	≥ 6.82	≥ 6.82	5.39	4.80	<3
G104H	<3	4.90	6.30	4.89	ND	<3
G106K	3.95	4.00	≥ 6.82	5.39	ND	<3
L107D	<b>6.39</b>	<3	3.00	3.40	ND	<3
I135S	≥ 6.82	≥ 6.82	≥ 6.82	5.39	ND	4.93
E138K	<b>6.82</b>	5.89	≥ 6.82	5.39	ND	3.43
T305E	4.39	6.40	≥ 6.82	5.39	5.40	5.03
T305H	5.39	6.40	≥ 6.82	5.39	5.86	5.03
E306G	5.09	6.40	ND	5.41	5.71	ND
K312E	4.91	5.39	≥ 6.82	5.39	<3	4.43
A315E	<3	4.60	≥ 6.82	5.39	<3	4.43
A315R	4.09	4.60	≥ 6.82	5.39	3.00	3.83
S329A	4.39	6.00	ND	5.41	3.60	ND
S331K	<3	5.39	ND	5.41	3.46	ND
G332R	<3	5.39	ND	5.11	4.20	ND
D389G	<3	4.60	ND	5.41	3.46	ND

\*Subgroup cross-reactive mAbs.

residues of EDIII can also be involved in the epitopes recognized by these mAbs.

The reactivity of seven JEV serocomplex cross-reactive mAbs, 2B5B-3, 6B4A-10, 16, 109, 203, 1B5D-1 and 7A6C-5, with WT JEV VLPs was demonstrated by end-point titres ranging from  $10^{3.95}$  to  $\geq 10^{6.82}$  (Table 3). Of amino acid substitutions located in EDII, W101G reduced reactivity with mAbs 2B5B-3, 6B4A-10 and 1B5D-1; G104H with mAb 16; and G106K with 2B5B-3 and 1B5D-1. Neither of the EDI amino acid substitutions affected the reactivity of the JEV serocomplex cross-reactive mAbs examined. The reactivity of mAbs 6B4A-10, 109 and 1B5D-1 was reduced by EDIII substitutions A315E and A315R; mAbs 203 and 1B5D-1 by K312E; and mAb 16 by T305H, S331K and D389G. Mutations L107D and T305E appeared to enhance the reactivity of mAb 6B4A-10, and mAb 16 was affected similarly by the W101K mutation. These results suggest that, for individual complex cross-reactive mAbs, residues in either EDII or EDIII can play critical roles in the epitopes recognized by these mAbs, and that for three mAbs (6B4A-10, 16 and 1B5D-1), there are epitope determinants in both EDII and EDIII.

### Cross-reactive epitopes on the JEV E protein

Based on the pattern of mAb reactivity with JEV WT and mutant VLPs, flavivirus group and JEV serocomplex

cross-reactive mAbs can be classified into five and six different epitope groups, respectively (Table 4). The differential patterns of substitutions in EDI, EDII and EDIII, acting as epitope determinants for the six flavivirus group cross-reactive mAbs examined here, suggest the existence of at least five overlapping yet distinct epitopes recognized by these antibodies. All group cross-reactive mAbs examined were affected by substitutions at amino acid W101. Similarly, G104, G106 and L107 appear to be incorporated into three group cross-reactive epitopes (Table 4; Fig. 1). The epitopes recognized by 4G2, 6B3B-3 and 6B6C-1 appear to additionally include different subsets of amino acids in EDIII; only one of the amino acid residues examined in EDI appears to be involved in any of the group cross-reactive mAb epitopes (5-2). These results suggest that the amino acid residues of the EDII fusion loop, and especially W101G, are most critical to group cross-reactive mAb epitopes, followed by residues of EDIII and then EDI (Table 4; Fig. 1).

The seven JEV serocomplex cross-reactive mAbs examined appeared to bind six different epitopes (Table 4). The fusion loop amino acids seem to be involved in four different JEV serocomplex cross-reactive epitopes, including those bound by mAbs 2B5B-3, 6B4A-10, 16 and 1B5D-1. However, W101 does not appear to play as critical a role in the binding of these complex cross-reactive mAbs as it

**Table 3.** Serocomplex cross-reactive mAb reactivity against WT and mutant VLPs

mAb end-point reactivity of WT and mutant VLPs was determined via Ag-ELISA and is expressed on a logarithmic scale. ND, Not determined. Underlining indicates a mutant VLP Ag-ELISA end-point titre reduced by >4-fold that of WT VLPs; bold type indicates a mutant VLP Ag-ELISA end-point titre increased by >4-fold that of WT VLPs.

VLPs	Complex cross-reactive mAbs (reciprocal log <sub>10</sub> end-point titre)						
	2B5B-3*	6B4A-10	16	109	203	1B5D-1†	7A6C-5†
WT	≥6.82	4.43	4.91	4.20	4.43	3.95	4.91
W101G	<u>5.86</u>	<u>&lt;3</u>	4.43	4.80	3.95	<u>&lt;3</u>	4.91
W101K	≥6.82	4.43	≥ <b>6.11</b>	4.80	4.80	4.55	4.91
W101E	≥6.82	4.43	5.51	4.80	4.80	4.55	4.91
W101F	≥6.82	4.43	5.51	4.80	4.43	4.55	5.51
G104H	6.30	3.93	<u>&lt;3</u>	ND	ND	3.35	ND
G106K	<u>&lt;3</u>	4.93	5.30	ND	ND	<u>&lt;3</u>	ND
L107D	≥6.82	<b>5.43</b>	5.30	ND	ND	4.45	ND
I135S	≥6.82	4.93	4.40	ND	ND	3.95	ND
E138K	≥6.82	4.93	4.40	ND	ND	3.95	ND
T305E	≥6.82	≥ <b>5.03</b>	4.91	4.20	4.80	3.95	4.91
T305H	≥6.82	3.83	<u>3.71</u>	3.60	4.43	3.35	4.91
E306G	6.30	ND	4.91	ND	ND	ND	4.91
K312E	≥6.82	4.91	4.91	4.20	<u>&lt;3</u>	<u>&lt;3</u>	5.39
A315E	≥6.82	<u>3.23</u>	4.31	<u>3.00</u>	4.43	<u>&lt;3</u>	4.91
A315R	≥6.82	<u>3.23</u>	4.31	<u>3.00</u>	4.43	<u>&lt;3</u>	4.91
S329A	6.30	ND	4.91	ND	ND	ND	4.91
S331K	6.30	ND	<u>4.20</u>	ND	ND	ND	4.91
G332R	6.30	ND	4.91	ND	ND	ND	4.91
D389G	6.30	ND	<u>4.20</u>	ND	ND	ND	4.91

\*Supercomplex cross-reactive mAbs.

†Subcomplex cross-reactive mAbs.

does for the group cross-reactive mAbs. The EDIII amino acid residues investigated seem to be involved in five different JEV serocomplex cross-reactive epitopes, affecting

**Table 4.** Amino acid residues in the E protein influencing group and serocomplex cross-reactive mAb recognition

GCR, Group cross-reactivity; CCR, complex cross-reactivity.

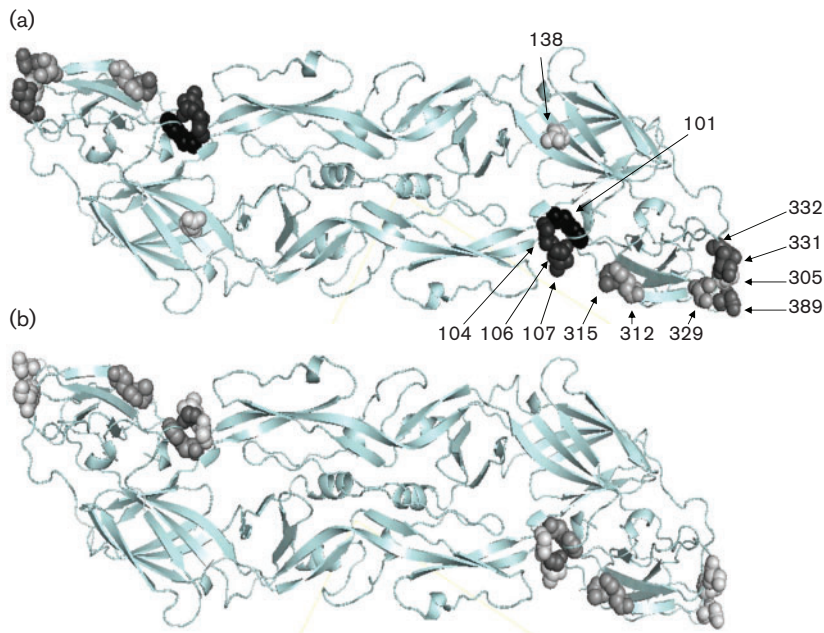
Class/ mAb	Domain residue		
	I	II	III
<b>GCR</b>			
4G2		101, 104, 106, 107	312, 315, 331, 332, 389
6B3B-3		101,	312, 315, 329, 331, 332, 389
6B6C-1		101, 104, 106	305, 315, 329, 331, 332, 389
23-1, 23-2		101, 107	
5-2	138	101, 104, 106, 107	
<b>CCR</b>			
2B5B-3		101, 106	
16		104	305, 331, 389
203			312
1B5D-1		101, 106	312, 315
6B4A-10		101	315
109			315

the reactivity of mAbs 6B4A-10, 16, 109, 203 and 1B5D-1. None of the six JEV serocomplex cross-reactive epitopes appeared to include the EDI amino acid residues investigated in this study. Also, none of the mutations introduced in this study influenced the binding of mAb 7A6C-5, suggesting that this mAb recognizes an epitope including residues beyond any of those examined in this study (Table 3). These results suggest that the amino acid residues of both EDII and EDIII are important in JEV serocomplex cross-reactive mAb epitopes (Table 4; Fig. 1).

## DISCUSSION

Flavivirus infections elicit protective antibody responses primarily against the E glycoprotein (Kuno, 2003). Murine mAb studies demonstrate that EDI contains predominantly type-specific, EDIII both type-specific and complex cross-reactive, and EDII predominantly broadly cross-reactive epitopes (Crill & Chang, 2004; Oliphant *et al.*, 2006; Roehrig *et al.*, 1983, 1989, 1990, 1998; Stiasny *et al.*, 2006). Here, we demonstrate that group cross-reactive epitopes are affected most dramatically by substitutions of the amino acid residues of the fusion loop of EDII, followed by residues of EDIII and EDI. Amino acid residues of both EDII and EDIII act as epitope determinants of JEV serocomplex cross-reactive mAbs.





**Fig. 1.** Structural locations of JEV E protein cross-reactive epitope residues. (a) Amino acid positions reactive with group cross-reactive mAbs; (b) amino acid positions reactive with serocomplex cross-reactive mAbs. Amino acid shading represents the number of mAb epitopes affected according to Table 4 (darkest, involved in five mAb epitopes; lightest, involved in one mAb epitope).

The importance of residues within the flavivirus FP as cross-reactive antigenic determinants has received attention (Crill & Chang, 2004; Crill *et al.*, 2007; Oliphant *et al.*, 2006; Stiasny *et al.*, 2006). Functional conservation across the flaviviruses in the FP region leads to the strong cross-reactive immunogenicity of this region. The fusion loop appears to be buried at the EDIII interface of the adjacent E protein that forms an E homodimer in the mature virion, seemingly precluding antibody binding to this region (Modis *et al.*, 2004; Nybakken *et al.*, 2006; Rey *et al.*, 1995; Stiasny *et al.*, 2006). However, decreases in dimer affinity within the virion, especially in different pH environments, could allow antibodies access to buried residues near the fusion loop. Previously published results predicted that EDIIFP residues W101, G104, G106 and L107 would be involved in flavivirus group cross-reactive epitopes (Chiou *et al.*, 2008; Crill & Chang, 2004; Crill *et al.*, 2007; Lai *et al.*, 2008). However, we are surprised that JEV serocomplex cross-reactive epitopes are also influenced by residues W101, G104, G106 and L107.

Compared with substitutions of the other conserved residues of the fusion loop, the W101G substitution dramatically reduces reactivity with all flavivirus group cross-reactive mAbs investigated. In support of the important role of W101 for flavivirus group cross-reactive antibody recognition, a recent study demonstrated that the predominantly cross-reactive polyclonal human sera generated during primary and secondary DENV infection recognizes epitopes that involve the W101 residue at the fusion loop of EDII (Lai *et al.*, 2008). Additionally, all of the broadly cross-reactive mAbs generated from WNV-infected mice that recognized the WT EDI–EDII tryptic E fragment exhibited significantly reduced binding for W101 mutant antigens (Oliphant *et al.*, 2006). Therefore, the W101

residue appears to be the fusion loop residue incorporated most frequently into the overlapping epitopes of antibodies recognizing this antigenic region.

Although the most critical amino acid residues of the group cross-reactive epitopes seem to be located in the fusion loop of EDII, the residues of EDIII also play an important role. On the mature virion, the E protein forms a structurally compact head-to-tail dimer (Modis *et al.*, 2004; Nybakken *et al.*, 2006; Rey *et al.*, 1995). A previous study suggested the involvement of the conserved residues 393–401 of EDIII in the binding of group cross-reactive mAbs (Falconar, 2008). Although we did not investigate substitutions in this region of the E protein, in this study we found several residues in EDIII, including T305, K312, A315, S329, S331, G332 and D389, that influenced three of five group cross-reactive epitopes. The well-characterized, overlapping epitopes of mAbs 4G2 and 6B6C-1 were affected by substitutions at S331 and G332, which in a previous study also influenced mAb binding of a JEV type-specific epitope, and at D389, which is involved in receptor binding (Chiou & Chen, 2007).

The DENV complex cross-reactive mAbs have been shown to recognize a set of overlapping epitopes that form a single antigenic site on the lateral surface of domain III, consisting of residues V305, L306, K308, E309, V310, K325, G381, D382, A384, K386, I387, W389 and R391 (Matsui *et al.*, 2009). In this study, we characterized six JEV complex cross-reactive epitopes by identifying amino acid residues of both EDII and EDIII that influence the binding of several JEV serocomplex cross-reactive mAbs. However, we did not identify a single most critical residue in the JEV serocomplex cross-reactive epitopes, analogous to W101 in flavivirus group-reactive epitopes.

Major conformational changes induced by these substitutions are unlikely because end-point titres of polyclonal murine hyperimmune ascitic fluid (MHIAF) are very similar between these mutants and WT JEV VLPs, although minor conformational alterations cannot be ruled out. Nevertheless, the mean area buried by the recognition of antibody–antigen complex is approximately  $1600 (\pm 400) \text{ \AA}^2$  (Lo Conte *et al.*, 1999). In this study, the longest distance between the EDIIFP and the EDIII residues (G104 and D389) recognized by mAbs 4G2, 6B6C-1 and 16 (Table 4) is approximately  $44.1 \text{ \AA}$  between domains within an E dimer or  $22.6 \text{ \AA}$  between domains across adjacent E dimers of the mature virion. Moreover, in the context of adjacent immature E trimers and dimers in partially mature virions, the distance between such residues is unknown, but it could be even closer than our estimation. Thus, it is likely that these residues, acting as epitope determinants for these cross-reactive mAbs, can be accommodated within the footprint of a single mAb. Additionally, mutations reducing E-specific mAb recognition do not appear to be due to the alteration of prM cleavage, processing or assembly, or VLP structure. prM processing, determined by the furin cleavage site between pr and M, is not affected by the E mutation, because, compared with WT VLP, the seven VLP mutants expressed a similar ratio of E/prM (Fig. S1, available in JGV Online). Viable mutant viruses (E138K and D389G) were available from our previous study (Chiou & Chen, 2007). We performed Ag-ELISA using these two mutant viruses with selected mAbs. The results support that the potential structural difference between VLPs and infectious virus did not distort our results, as E138K virions reduced mAb 5-2 binding by 8-fold and D389G virions reduced mAb 4G2 and 16 binding by 32- and 4-fold, respectively (data not shown; Ferlenghi *et al.*, 2001).

Interestingly, none of the mutated JEV VLPs used in this study demonstrated reduced reactivity with JEV type-specific mAbs, whilst some experienced altered binding of flavivirus group-reactive and JEV serocomplex cross-reactive mAbs. Thus, the JEV type-specific epitopes probably do not overlap with flavivirus group cross-reactive and JEV serocomplex cross-reactive epitopes. Our previous report showed that using G106K/L107D double-mutant JEV VLPs as ELISA antigens had no effect on assay sensitivity, but dramatically increased the specificity in distinguishing acute JEV infection from other flavivirus infections in humans (Chiou *et al.*, 2008). Studies incorporating amino acid residue(s) of EDIII and W101G of EDII into G106K/L107D double-mutant JEV VLPs may greatly improve the specificity of antigens and vaccine design.

## METHODS

**VLP expression plasmid.** We used the recombinant expression plasmid pVAX-JEi as the template DNA for both site-directed mutagenesis and the transient expression of JEV WT recombinant

antigen (see below) (Chang *et al.*, 2000, 2007; Hunt *et al.*, 2001). The pVAX-JEi includes the human cytomegalovirus (CMV) early gene promoter, JEV signal sequence, JEV premembrane/membrane (prM/M) and E gene region in its entirety, intron, bovine growth hormone poly(A) signal and a kanamycin-resistance gene.

**Site-directed mutagenesis.** Site-specific mutations, as shown in Table 1, were introduced into the JEV E gene of the pVAX-JEi plasmid using a QuikChange Multi site-directed mutagenesis kit (Stratagene) according to the manufacturer's recommended protocols. Clones were sequenced across the entire prM–E region to identify the correct mutant. Automated DNA sequencing was performed with an ABI 3130xl genetic analysis system (Applied Biosystems) and sequences were analysed with Lasergene software (DNASTAR).

**WT and mutant VLP expression.** COS-1 cells (ATCC CRL 1650; ATCC) were grown at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$  in Dulbecco's modified Eagle's minimal essential medium (Gibco) supplemented with 10% heat-inactivated FBS (HyClone Laboratories, Inc.). For transformation,  $0.5 \text{ ml}$  COS-1 cell suspension ( $1.5 \times 10^7 \text{ cells ml}^{-1}$ ) was electroporated with  $20 \mu\text{g}$  plasmid DNA in a  $0.4 \text{ cm}$  electrode gap cuvette with a Bio-Rad Gene Pulser II set at  $250 \text{ V}$  and  $975 \mu\text{F}$ . The transformed COS-1 cells were seeded into individual  $75 \text{ cm}^2$  culture flasks containing  $50 \text{ ml}$  growth medium and allowed to recover at  $37^\circ\text{C}$  overnight, after which time they were continually maintained at  $28^\circ\text{C}$  in serum-free medium (SFM4MegaVir; HyClone). Tissue-culture medium was harvested every 3 days, clarified by centrifugation at  $10\,000 \text{ r.p.m.}$  for  $30 \text{ min}$  at  $4^\circ\text{C}$  in a F-16/250 rotor (Beckman Coulter) and stored at  $4^\circ\text{C}$  for further analysis. The selected mutant VLPs were concentrated by centrifugation at  $19\,000 \text{ r.p.m.}$  for  $16 \text{ h}$  in a type 19 rotor (Beckman Coulter), resuspended in 1:100 or original volume, further purified by centrifugation at  $39\,000 \text{ r.p.m.}$  for  $4 \text{ h}$  in a SW41 rotor (Beckman Coulter) and resuspended in the same volume of  $1 \times \text{TNE}$  buffer ( $50 \text{ mM}$  Tris,  $140 \text{ mM}$  NaCl,  $5 \text{ mM}$  EDTA). The E/prM ratios for the selected WT and mutant VLPs were estimated by Western blot with a 1:500 dilution of JEV-infected swine serum obtained from our previous study. The estimated E/prM ratio was used to determine the potential influence of mutations on prM processing and maturation (Fig. S1).

In order to map the group and complex cross-reactive epitopes on the JEV E glycoprotein, amino acid substitutions were introduced into a WT JEV prM/E protein expression plasmid that directs the assembly of VLPs when transfected into mammalian cell culture. Substitutions at residues that were chosen based on previous reports or biological function were introduced into each of the three domains of the JEV E protein, and included I135 and E138 of EDI; W101, G104, G106 and L107 of EDII; and T305, E306, K312, A315, S329, S331, G332 and D389 of EDIII (Table S1). WT and mutant plasmids were transformed into COS-1 cells for VLP expression and the cells were incubated at  $28^\circ\text{C}$  to increase VLP secretion into culture supernatant. End points for tissue-culture fluid containing secreted JEV VLPs ranged from 1:32 to 1:4096 when titrated in an Ag-ELISA. Secreted antigen concentrations were standardized for mAb screening by selecting the antigen concentration producing an  $A_{450}$  value of approximately 1.0 with polyclonal anti-JEV MHIAF, which was equivalent to  $40 \text{ ng}$  20% sucrose cushion-purified VLPs (data not shown).

**mAb panel.** When selecting mAbs for use in antigen characterization, we specifically chose a variety of group cross-reactive, JEV serocomplex cross-reactive, and JEV type-specific mAbs that had been raised against a diverse assortment of flaviviruses (Gentry *et al.*, 1982; Kimura-Kuroda & Yasui, 1983, 1986; Roehrig *et al.*, 1983). mAbs 4G2, 23-1, 23-2, 6B3B-3, 5-2 and 6B6C-1 are flavivirus group cross-reactive (recognizing viruses from all major pathogenic

serocomplexes of flaviviruses) and are non- to weakly NT. mAbs 16, 6B4A-10, 1B5D-1, 109, 203, 2B5B-3 and 7A6C-5 exhibit various levels of cross-reactivity with viruses within the JEV serocomplex and are non-NT as well. J3 14 H5-2, 112, 2F2, 2H4 and 503 are the JEV-specific mAbs used in this study (Kimura-Kuroda & Yasui, 1986).

**mAb reactivity with VLPs.** The panel of mAbs was used to determine end points of mAb reactivity with mutated and WT antigens in Ag-ELISA as described previously (Crill & Chang, 2004). Secreted antigen concentrations were standardized for mAb screening by selecting the antigen concentration producing an  $A_{450}$  value of approximately 1.0 with polyclonal anti-JEV MHIAF, which was equivalent to 40 ng 20% sucrose cushion-purified VLPs (data not shown). Briefly, Immulon II HB flat-bottomed 96-well plates (Dynatech Industries, Inc.) were coated with rabbit anti-JEV hyperimmune serum using coating buffer (0.015 M sodium carbonate, 0.035 M sodium bicarbonate, pH 9.6) and incubated overnight at 4 °C. Wells were blocked with 300 µl StartingBlock (PBS) blocking buffer (Pierce). WT and mutant antigens were captured during a 1 h incubation at 37 °C. Plates were subsequently washed three times. mAbs were diluted serially in 5% milk, and 50 µl was added to the wells before incubation at 37 °C for 1 h. Plates were washed three times, and then mAbs were detected with HRP-conjugated goat anti-MHIAF at a 1:5000 dilution in PBS containing 5% milk. Bound conjugate was detected by adding 75 µl 3,3',5,5'-tetramethylbenzidine (TMB; Neogen Corp.) substrate and incubating at room temperature for 10 min. The substrate reaction was stopped with 50 µl 1 M  $H_2SO_4$ , and  $A_{450}$  was measured with an EL 312e Bio-Kinetics microplate reader (Bio-Tek Instruments, Inc.). The end-point titre of mAb reactivity was confirmed by repeat titration.

**JEV cross-reactive epitopes.** A homology model for the JEV E protein was produced using the published atomic coordinates for DENV-2 and TBEV and the SWISS-MODEL workspace (<http://swissmodel.expasy.org/workspace/>). Preferred potential epitopes were identified by Ag-ELISA using mutant JEV VLPs mapped onto an E dimer.

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## REFERENCES

- Beasley, D. W. & Barrett, A. D. (2002). Identification of neutralizing epitopes within structural domain III of the West Nile virus envelope protein. *J Virol* **76**, 13097–13100.
- Casals, J. & Brown, L. V. (1954). Hemagglutination with arthropod-borne viruses. *J Exp Med* **99**, 429–449.
- Chambers, T. J., Hahn, C. S., Galler, R. & Rice, C. M. (1990). Flavivirus genome organization, expression, and replication. *Annu Rev Microbiol* **44**, 649–688.
- Chang, G. J., Hunt, A. R. & Davis, B. (2000). A single intramuscular injection of recombinant plasmid DNA induces protective immunity and prevents Japanese encephalitis in mice. *J Virol* **74**, 4244–4252.
- Chang, G. J., Davis, B. S., Stringfield, C. & Lutz, C. (2007). Prospective immunization of the endangered California condors (*Gymnogyps californianus*) protects this species from lethal West Nile virus infection. *Vaccine* **25**, 2325–2330.
- Chiou, S. S. & Chen, W. J. (2007). Phenotypic changes in the Japanese encephalitis virus after one passage in Neuro-2a cells: generation of attenuated strains of the virus. *Vaccine* **26**, 15–23.
- Chiou, S. S., Crill, W. D., Chen, L. K. & Chang, G. J. (2008). Enzyme-linked immunosorbent assays using novel Japanese encephalitis virus antigen improve the accuracy of clinical diagnosis of flavivirus infections. *Clin Vaccine Immunol* **15**, 825–835.
- Crill, W. D. & Chang, G. J. (2004). Localization and characterization of flavivirus envelope glycoprotein cross-reactive epitopes. *J Virol* **78**, 13975–13986.
- Crill, W. D., Trainor, N. B. & Chang, G. J. (2007). A detailed mutagenesis study of flavivirus cross-reactive epitopes using West Nile virus-like particles. *J Gen Virol* **88**, 1169–1174.
- Crill, W. D., Hughes, H. R., Delorey, M. J. & Chang, G. J. (2009). Humoral immune responses of dengue fever patients using epitope-specific serotype-2 virus-like particle antigens. *PLoS One* **4**, e4991.
- Falconar, A. K. (2008). Use of synthetic peptides to represent surface-exposed epitopes defined by neutralizing dengue complex- and flavivirus group-reactive monoclonal antibodies on the native dengue type-2 virus envelope glycoprotein. *J Gen Virol* **89**, 1616–1621.
- Ferlenghi, I., Clarke, M., Ruttan, T., Allison, S. L., Schlich, J., Heinz, F. X., Harrison, S. C., Rey, F. A. & Fuller, S. D. (2001). Molecular organization of a recombinant subviral particle from tick-borne encephalitis virus. *Mol Cell* **7**, 593–602.
- Gentry, M. K., Henchal, E. A., McCown, J. M., Brandt, W. E. & Dalrymple, J. M. (1982). Identification of distinct antigenic determinants on dengue-2 virus using monoclonal antibodies. *Am J Trop Med Hyg* **31**, 548–555.
- Gromowski, G. D., Barrett, N. D. & Barrett, A. D. (2008). Characterization of dengue virus complex-specific neutralizing epitopes on envelope protein domain III of dengue 2 virus. *J Virol* **82**, 8828–8837.
- Halstead, S. B. (1988). Pathogenesis of dengue: challenges to molecular biology. *Science* **239**, 476–481.
- Hunt, A. R., Cropp, C. B. & Chang, G. J. (2001). A recombinant particulate antigen of Japanese encephalitis virus produced in stably-transformed cells is an effective noninfectious antigen and subunit immunogen. *J Virol Methods* **97**, 133–149.
- Kanai, R., Kar, K., Anthony, K., Gould, L. H., Ledizet, M., Fikrig, E., Marasco, W. A., Koski, R. A. & Modis, Y. (2006). Crystal structure of west nile virus envelope glycoprotein reveals viral surface epitopes. *J Virol* **80**, 11000–11008.
- Kimura-Kuroda, J. & Yasui, K. (1983). Topographical analysis of antigenic determinants on envelope glycoprotein V3 (E) of Japanese encephalitis virus, using monoclonal antibodies. *J Virol* **45**, 124–132.
- Kimura-Kuroda, J. & Yasui, K. (1986). Antigenic comparison of envelope protein E between Japanese encephalitis virus and some other flaviviruses using monoclonal antibodies. *J Gen Virol* **67**, 2663–2672.
- Kuno, G. (2003). Serodiagnosis of flaviviral infections and vaccinations in humans. *Adv Virus Res* **61**, 3–65.
- Lai, C. Y., Tsai, W. Y., Lin, S. R., Kao, C. L., Hu, H. P., King, C. C., Wu, H. C., Chang, G. J. & Wang, W. K. (2008). Antibodies to envelope glycoprotein of dengue virus during the natural course of infection are predominantly cross-reactive and recognize epitopes containing highly conserved residues at the fusion loop of domain II. *J Virol* **82**, 6631–6643.
- Lanciotti, R. S., Roehrig, J. T., Deubel, V., Smith, J., Parker, M., Steele, K., Crise, B., Volpe, K. E., Crabtree, M. B. & other authors (1999). Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. *Science* **286**, 2333–2337.
- Lindenbach, B. D. & Rice, C. M. (2003). Molecular biology of flaviviruses. *Adv Virus Res* **59**, 23–61.



- Lo Conte, L., Chothia, C. & Janin, J. (1999).** The atomic structure of protein-protein recognition sites. *J Mol Biol* **285**, 2177–2198.
- Mackenzie, J. S., Gubler, D. J. & Petersen, L. R. (2004).** Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. *Nat Med* **10** (Suppl.), S98–S109.
- Matsui, K., Gromowski, G. D., Li, L., Schuh, A. J., Lee, J. C. & Barrett, A. D. (2009).** Characterization of dengue complex-reactive epitopes on dengue 3 virus envelope protein domain III. *Virology* **384**, 16–20.
- Modis, Y., Ogata, S., Clements, D. & Harrison, S. C. (2004).** Structure of the dengue virus envelope protein after membrane fusion. *Nature* **427**, 313–319.
- Nybakken, G. E., Nelson, C. A., Chen, B. R., Diamond, M. S. & Fremont, D. H. (2006).** Crystal structure of the West Nile virus envelope glycoprotein. *J Virol* **80**, 11467–11474.
- Oliphant, T., Nybakken, G. E., Engle, M., Xu, Q., Nelson, C. A., Sukupolvi-Petty, S., Marri, A., Lachmi, B. E., Olshevsky, U. & other authors (2006).** Antibody recognition and neutralization determinants on domains I and II of West Nile Virus envelope protein. *J Virol* **80**, 12149–12159.
- Rey, F. A., Heinz, F. X., Mandl, C., Kunz, C. & Harrison, S. C. (1995).** The envelope glycoprotein from tick-borne encephalitis virus at 2 Å resolution. *Nature* **375**, 291–298.
- Roehrig, J. T., Mathews, J. H. & Trent, D. W. (1983).** Identification of epitopes on the E glycoprotein of Saint Louis encephalitis virus using monoclonal antibodies. *Virology* **128**, 118–126.
- Roehrig, J. T., Hunt, A. R., Johnson, A. J. & Hawkes, R. A. (1989).** Synthetic peptides derived from the deduced amino acid sequence of the E-glycoprotein of Murray Valley encephalitis virus elicit antiviral antibody. *Virology* **171**, 49–60.
- Roehrig, J. T., Johnson, A. J., Hunt, A. R., Bolin, R. A. & Chu, M. C. (1990).** Antibodies to dengue 2 virus E-glycoprotein synthetic peptides identify antigenic conformation. *Virology* **177**, 668–675.
- Roehrig, J. T., Bolin, R. A. & Kelly, R. G. (1998).** Monoclonal antibody mapping of the envelope glycoprotein of the dengue 2 virus, Jamaica. *Virology* **246**, 317–328.
- Stiasny, K., Kiermayr, S., Holzmann, H. & Heinz, F. X. (2006).** Cryptic properties of a cluster of dominant flavivirus cross-reactive antigenic sites. *J Virol* **80**, 9557–9568.
- Sukupolvi-Petty, S., Austin, S. K., Purtha, W. E., Oliphant, T., Nybakken, G. E., Schlesinger, J. J., Roehrig, J. T., Gromowski, G. D., Barrett, A. D. & other authors (2007).** Type- and subcomplex-specific neutralizing antibodies against domain III of dengue virus type 2 envelope protein recognize adjacent epitopes. *J Virol* **81**, 12816–12826.
- Trainor, N. B., Crill, W. D., Roberson, J. A. & Chang, G. J. (2007).** Mutation analysis of the fusion domain region of St. Louis encephalitis virus envelope protein. *Virology* **360**, 398–406.
- Vaughn, D. W. & Hoke, C. H., Jr (1992).** The epidemiology of Japanese encephalitis: prospects for prevention. *Epidemiol Rev* **14**, 197–221.